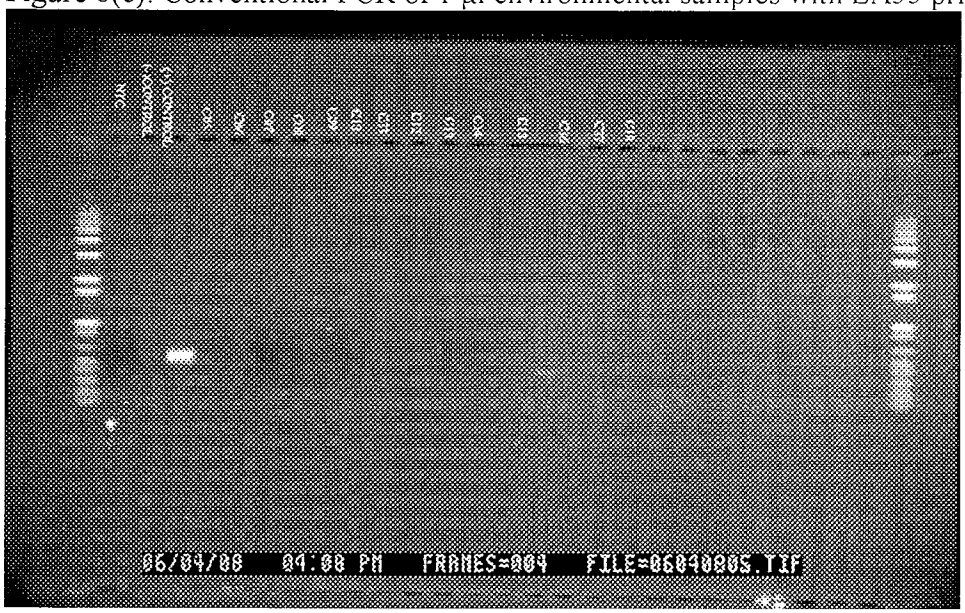
Figure 8(c): Conventional PCR of 1 µl environmental samples with LA35 primers



Results in Figures 8a-c show that only a small number of environmental DNA samples produced fragments after PCR. To determine if the lack of PCR product was due to either a true negative (i.e. lack of the *Brevibacterium* LA35 forward or reverse sites) or due to inhibition, the environmental samples were amplified with a set of universal bacterial 16S rDNA primers. The 8F and 907R primer set were used in this reaction, and 10 ng of each DNA sample was amplified in each reaction. The PCR protocol is the same as that used earlier for conventional PCR with the LA35 primers. Results of these studies are show in Figures 9a-c.

Figure 9(a): Conventional PCR with universal bacterial 16S rRNA primers

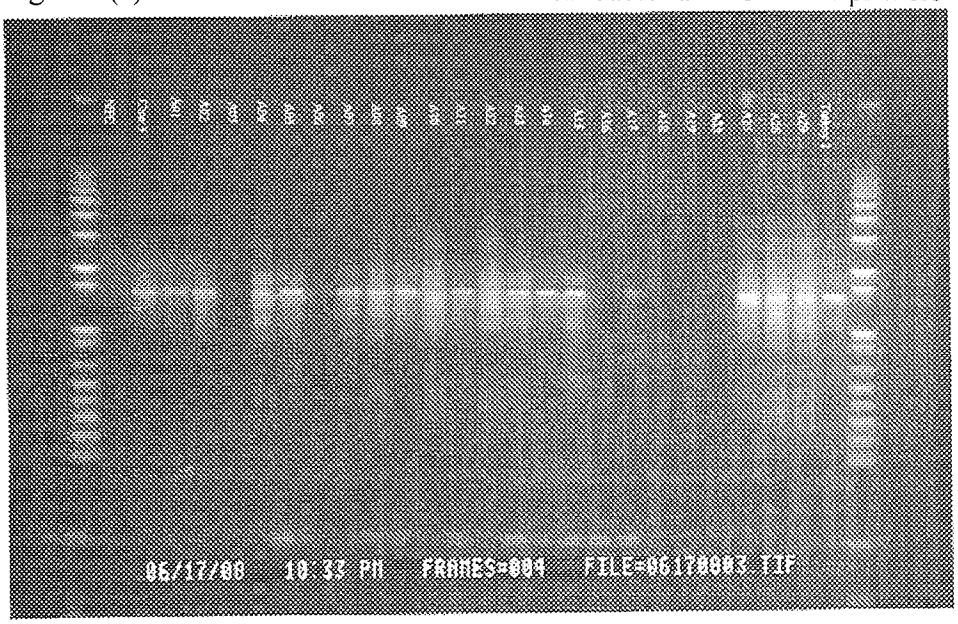


Figure 9(b): Conventional PCR with universal bacterial 16S rRNA PCR primers

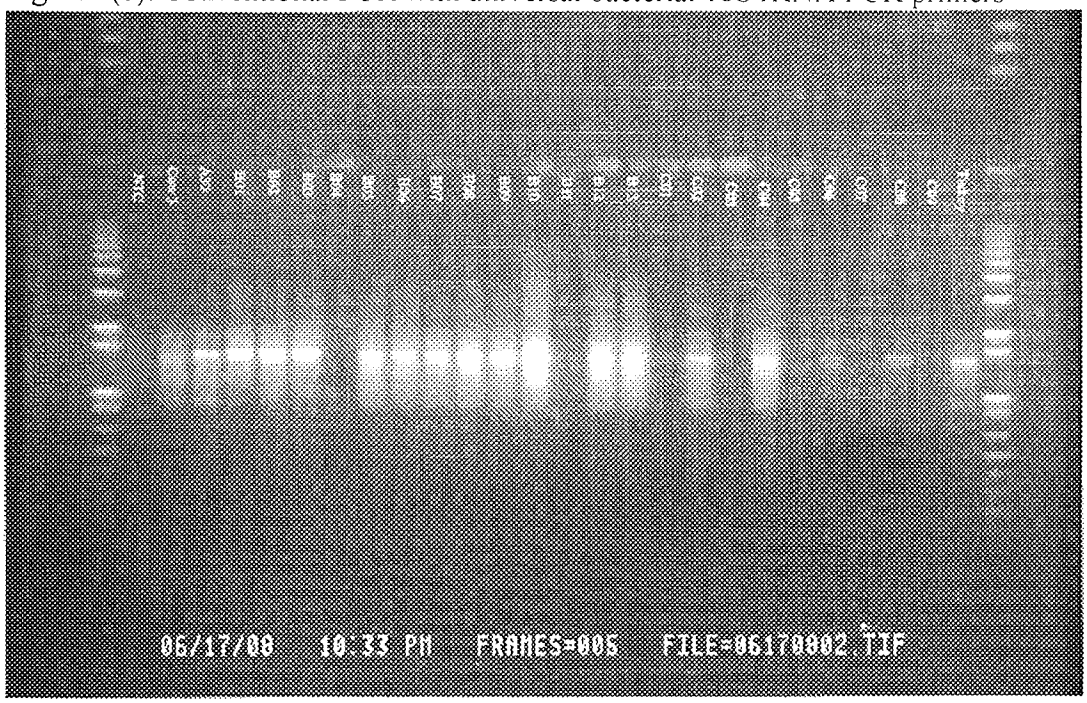
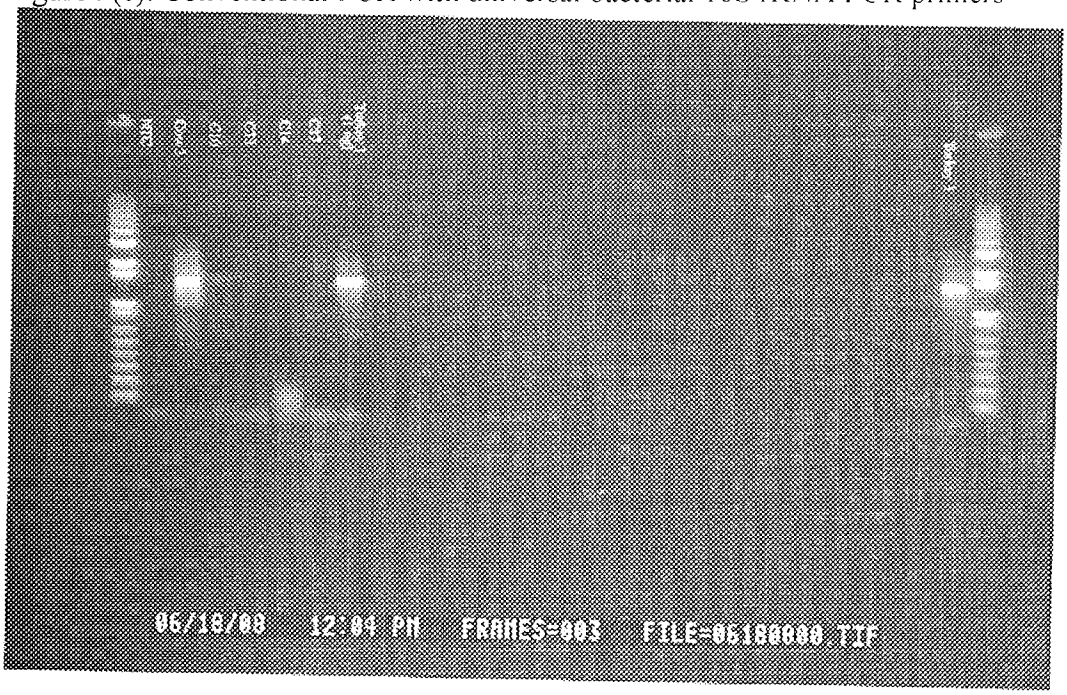


Figure 9(c): Conventional PCR with universal bacterial 16S rRNA PCR primers



Since all samples contained the same quantity of DNA, the negative results in the experiment with the universal 16S rDNA PCR primers suggest that there must have been inhibitors present that interfered with PCR. To test to see if there were any inhibitors present, and to find the optimum DNA concentration to use to avoid inhibition, the PCR assays with universal PCR primers were repeated with two lower (1 and 5 ng) and one higher concentration (25 ng) of

template DNA from environmental samples in each reaction. Furthermore, a matrix spike experiment was run where 10 ng of the DNAs from the environmental samples were added to 10 ng of DNA extracted from turkey feces and the mixture was amplified with universal 16S rDNA primers. In a matrix spike experiment, samples which are suspected to contain an inhibitor are added to a positive control which should always give product in the absence of inhibition. If the positive control added to the matrix spike does not give a product, it is due to the presence of an inhibitor.

Figure 10: PCR with universal bacterial 16S rRNA primers at a 1 ng DNA concentration

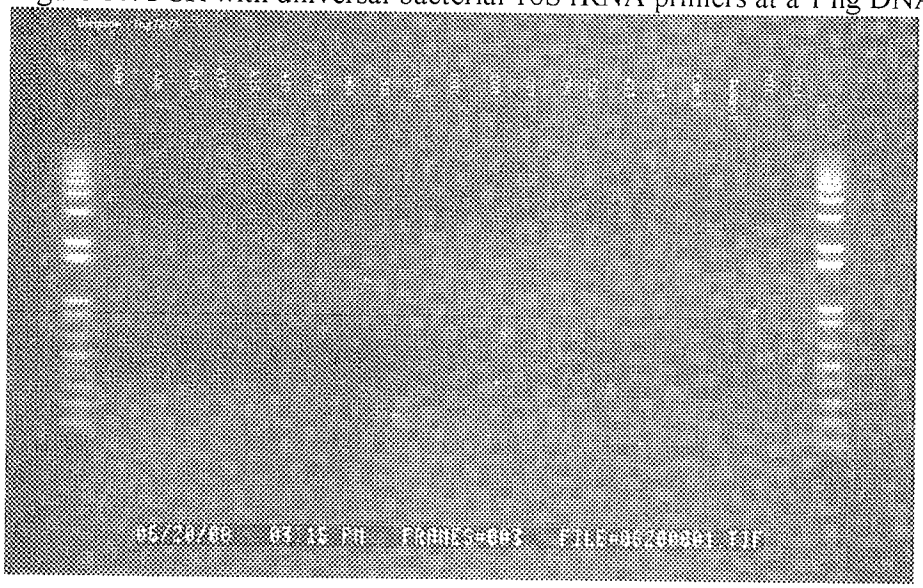


Figure 11: PCR with universal primers at a 5ng DNA concentration

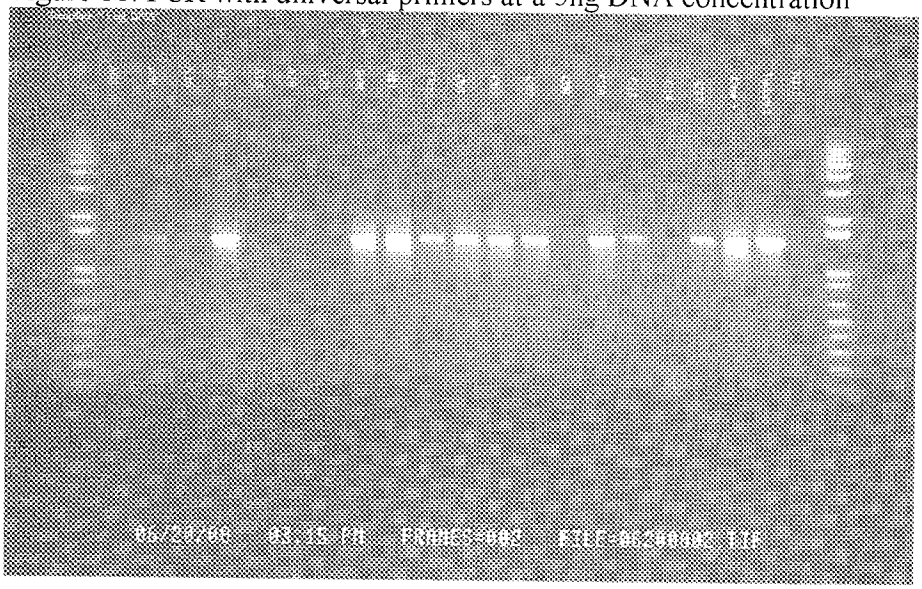


Figure 12: PCR with universal primers at a 25ng DNA concentration

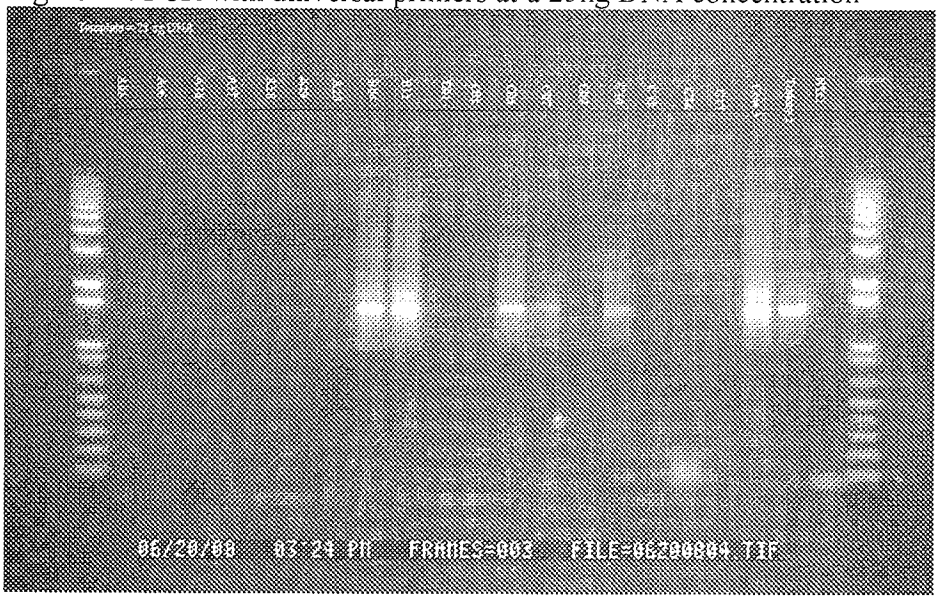
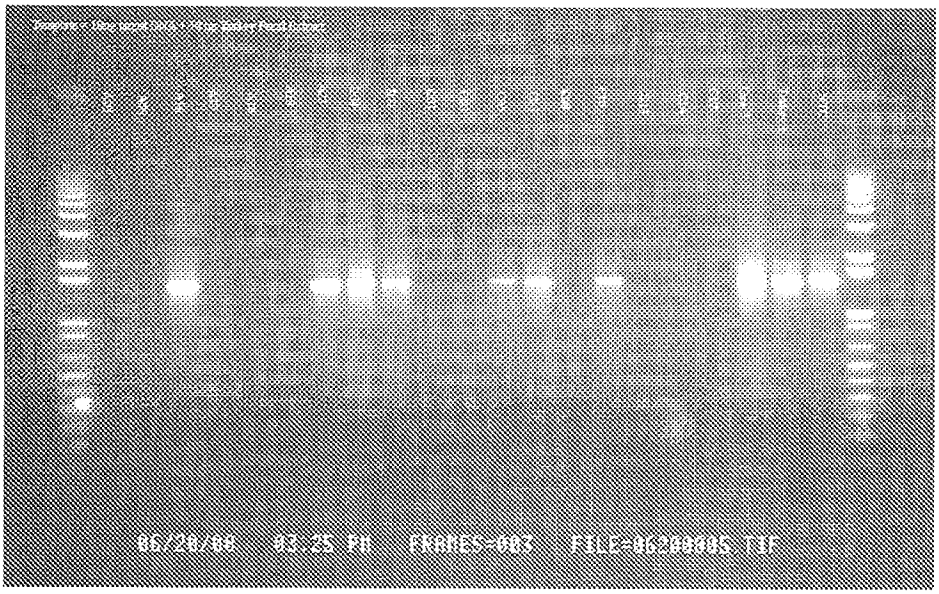


Figure 13: Matrix spike PCR with universal primers using 10ng of DNA from environmental extracts and 10 ng of DNAs from Turkey fecal extracts.



Results of these studies (Figures 10-13) indicated that: 1) a threshold concentration of 5 ng of DNA is needed to give a PCR product using 16S rDNA universal primers, that 2) raising the DNA concentration to 25 ng inhibited some reactions, and 3) based on the matrix spike experiment, many of the samples that gave no PCR products in the PCR with LA35 primer, likely contained inhibitors that interfered with the PCR reaction. This included samples: A03, A06, A17, A18, A19, C01, C03, C09, C13, C14 and C17. Of the DNA concentrations tested using conventional PCR, 5 ng DNA per 25 µl reaction gave the most samples with product among previous negative samples. Consequently, 5 ng of DNAs from environmental samples were used for all further PCR and qPCR reactions, unless otherwise noted.

Results in Figures 14a-14c show that repeating the above PCR with LA35 primers using 5 ng of DNA instead of the suggested 15 ng, produced results that more closely coincided with those obtained using the universal 16S primers on 6/16/2008. These results showed that the entirety of the "B" set of isolates were positive for the poultry fecal marker. In contrast, from sets "A" & "C", only samples A33 and C01 were positive by PCR, and showed strong signals. However, several of the A samples (A25, 27, 28, 28, 29 and 31) showed smaller amounts of product following PCR.

Figure 14(a): Conventional PCR of 5 ng environmental samples with LA35 primers

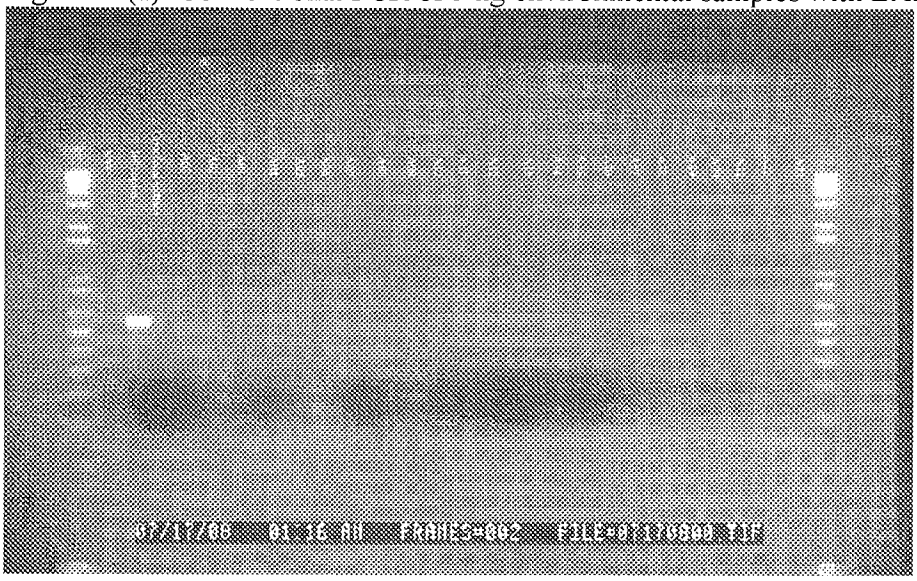


Figure 14(b): Conventional PCR of 5 ng environmental samples with LA35 primers

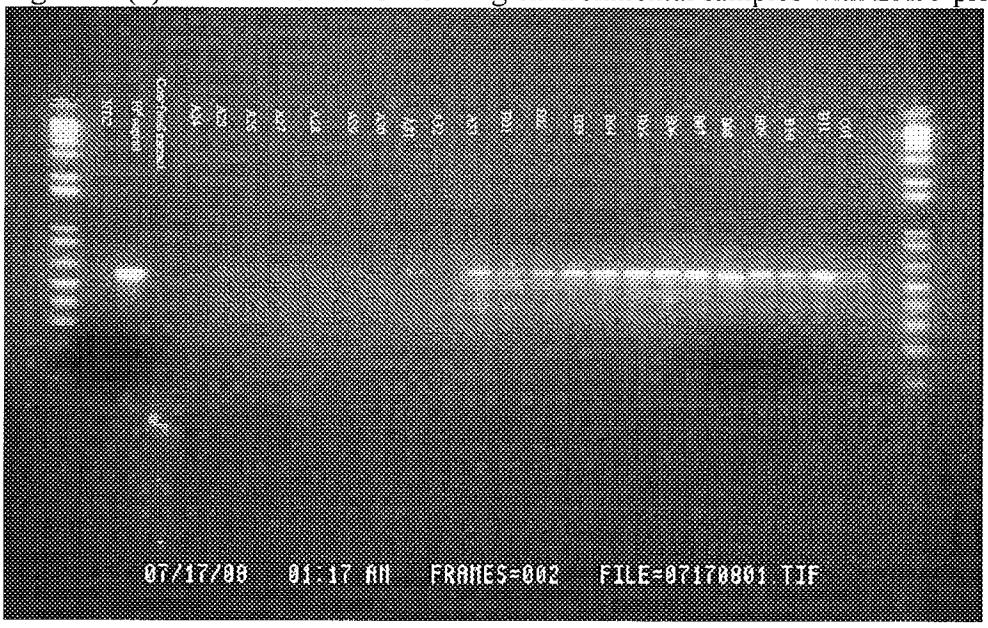
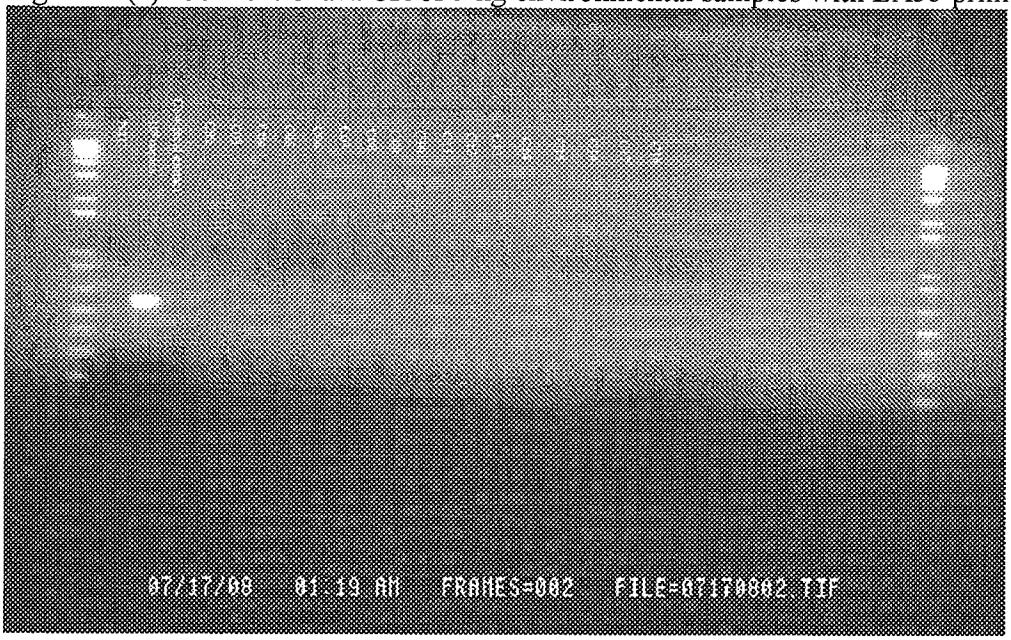


Figure 14(c): Conventional PCR of 5 ng environmental samples with LA35 primers



IV. Optimum annealing temperature

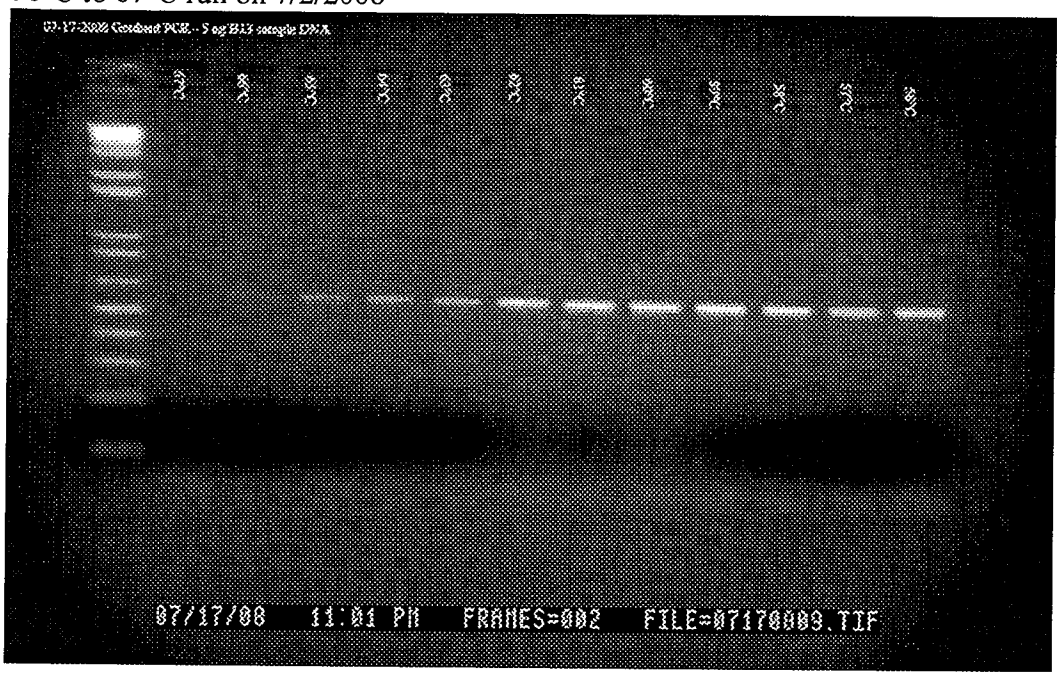
After determining the concentration of sample DNA to use and which samples contained inhibitors, we attempted to optimize the PCR protocol with regards to the annealing temperature. This was done to maximize band intensity and reduce primer dimers. All experiments were performed on a Stratagene Robocycler gradient 96 thermal cycler.

A temperature gradient PCR was performed using the same reagents as were used for conventional PCR with the LA35 primer set and the poultry litter biomarker plasmid as a template. For each 25 µl reaction, 5 µg of template DNA was added to 1 x Promega clear GoTaq PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM each of the forward and reverse LA35 primers, 0.02 U per µl and sterile water to 24 µl.

The thermal cycler protocol was changed from those used for the previous PCR assays since the gradient thermal cycler lags in heating and cooling samples when compared to a normal thermal cycler. While the initial incubations of 5 minute incubation at 94°C stayed the same, the amplification was changed to 40 cycles of 45 seconds of dissociation at 94°C, 45 seconds of annealing at 56 to 67°C, and 2 minute and 5 seconds of elongation at 72°C. This protocol was followed by a 7 minute final elongation at the matching annealing temperature, from 56 to 67°C and held at 4°C.

Results in Figure 15 done using conventional gradient PCR indicated that the suggested annealing temperature of 60°C was adequate to amplify the PCR fragment of interest.

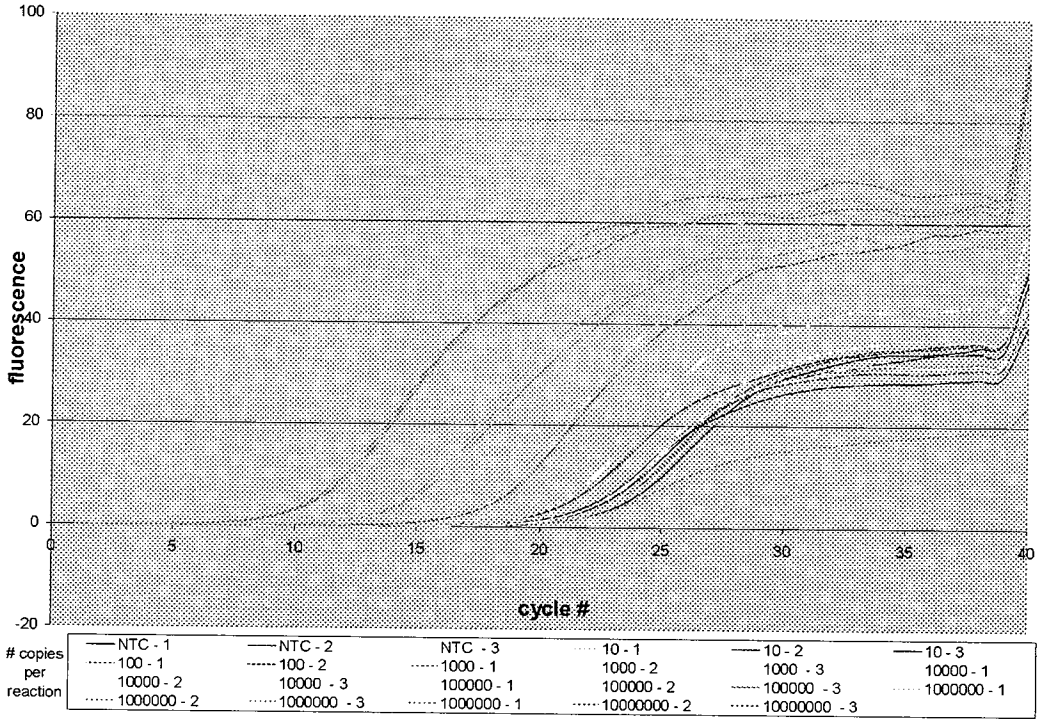
Figure 15: Gradient PCR on the poultry litter biomarker plasmid in the range of temperatures 56°C to 67°C run on 7/2/2008



V. Increasing the temperature of the elongation step to avoid formation of secondary products

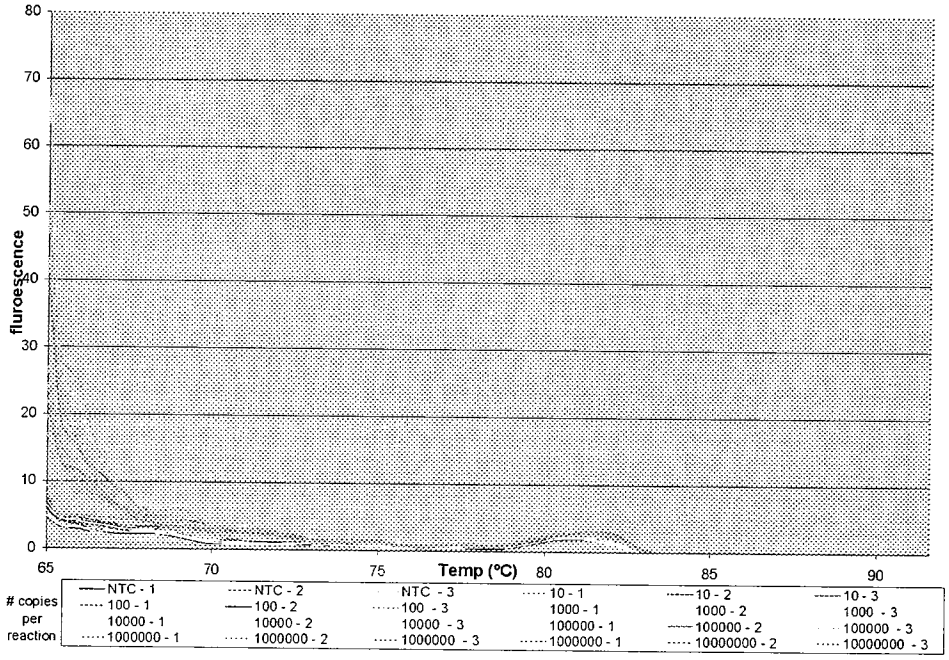
Since the above protocol now giving proper sigmoid fluorescence curves (Figure 4), an experiment was done to generate a standard curve using the control plasmid DNA. Results in Figure 16 show that while the curves are properly spaced in relation to each other with regards to product formed in relation to DNA concentration, the controls with a low copy number of target sequence were crossing the threshold Ct value too early in the reaction. For example in the experiment performed on 7/3/08, the non-template control had a Ct value between 22 and 23 cycles, suggesting that another, spurious product was causing fluorescence.

Figure 16: Quantitative PCR fluorescence curve generated from poultry litter biomarker template and LA35 primer set for 7/03/2008



To determine what was causing the low Ct (non-specific fluorescence) for the non-template sample, we looked at the melting curve for the reaction, which measures the change in fluorescence with increasing temperature.

Figure 17: Melting curve for 7/03/2008



Results in Figure 17 show that a secondary product was generated by PCR between the temperatures of 65 to 70°C. While the correct PCR product of interest occurs at a peak near 80°C, we were concerned that the secondary product may have been a primer dimer pair (spontaneous self-annealing of primers) and that this could have reduced the effective primer concentration needed to give adequate products for qPCR. If so, one could expect nonspecific fluorescence in samples without template DNA, such as blanks, and one could also expect to see reduced sensitivity to low copy numbers of target DNA.

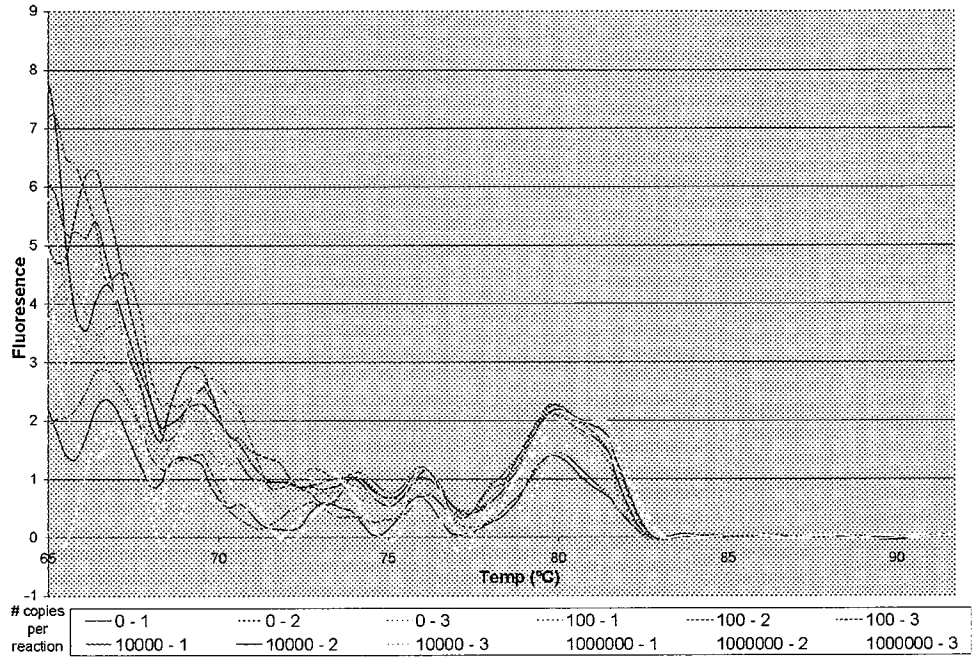
To examine this issue in more detail, the LA35 primer set was analyzed by using the Premier Biosoft NetPrimer software (http://www.premierbiosoft.com/netprimer/index.html). The free energy of binding of the actual primers (ΔG) was -33.23 kcal/mol. There was a primer dimer of the forward primer (Figure 19) which has a free energy of the same order of magnitude of this energy, ΔG=-9.75 kcal/mol. This suggested that primer dimmers may be impacting the qPCR reaction. The goal of the next set of experiments was to prevent to production of this dimer.

Figure 18: Dimer of LA35F primer

```
              5' ACCGGATACGACCATCTGC 3'
                   ||||
3' CGTCTACCAGCATAGGCCA 5'
```

With the problem being one of poor amplification and generation of nonspecific secondary products, the primer concentration was varied to try and increase the amount of specific product and limit the amount of primer available to make byproducts. The first attempt was to increase primer concentration from the suggested 0.5 μM final concentration to a 1 μM concentration. Figure 19 shows that an increasing primer concentration increased the relative height in the product region of the melt curve compared to the secondary product region, but did not change the low Ct value for the background non-template control sample, at 22.5 cycles.

Figure 19: Dissociation curve for 7/06/2008 data.



Since increasing primer concentration did not appear to address this issue, the influence of lower primer concentration on Ct values and primer dimer formation was investigated. Decreasing primer concentration should decrease the amount of errant (unbound) primers available for the creation of primer dimers. The concentrations of primers tested were 500, 250, 125, and 62.5 nM (500 nM is equivalent to 0.5 μM). Results of these studies (Figures 20-23) indicated that a decrease in primer concentration caused an increase in the ratio of product to dimer, while keeping the Ct value for the negative control (water) at or above the desired 30 cycles cut-off. This was an improvement.

Figure 20: Dissociation curve for PCR performed on 7/7/2008 with 500 nM primer conc.

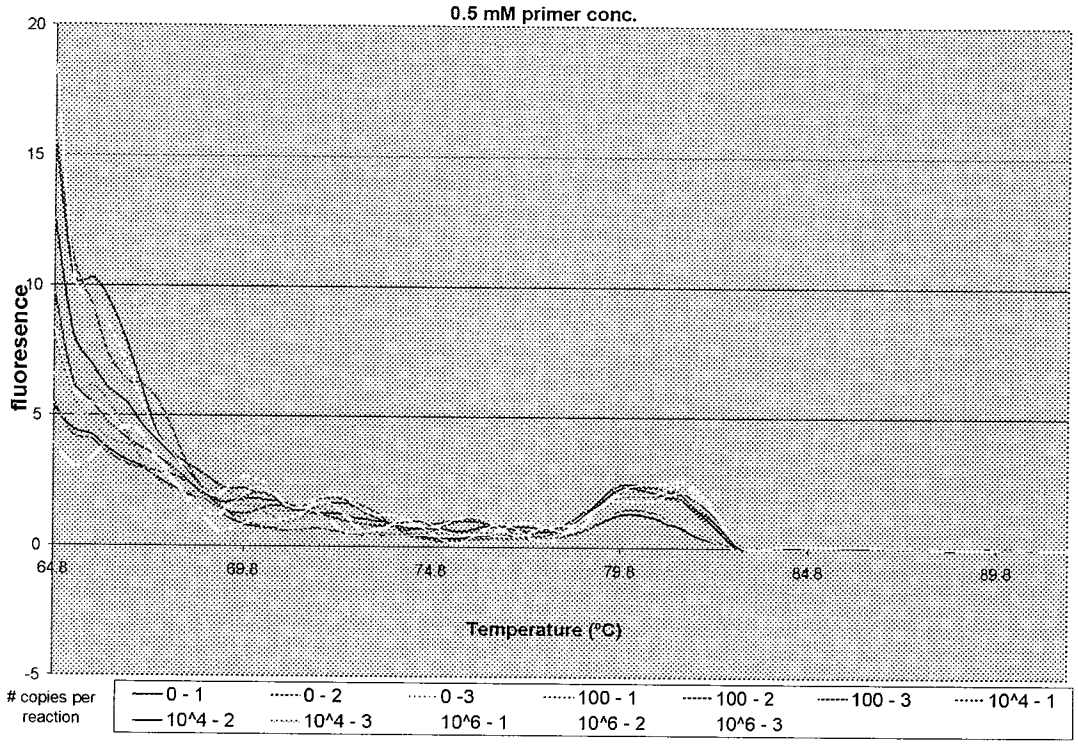


Figure 21: Dissociation curve for PCR performed on 7/7/2008 with 250 nM primer conc.

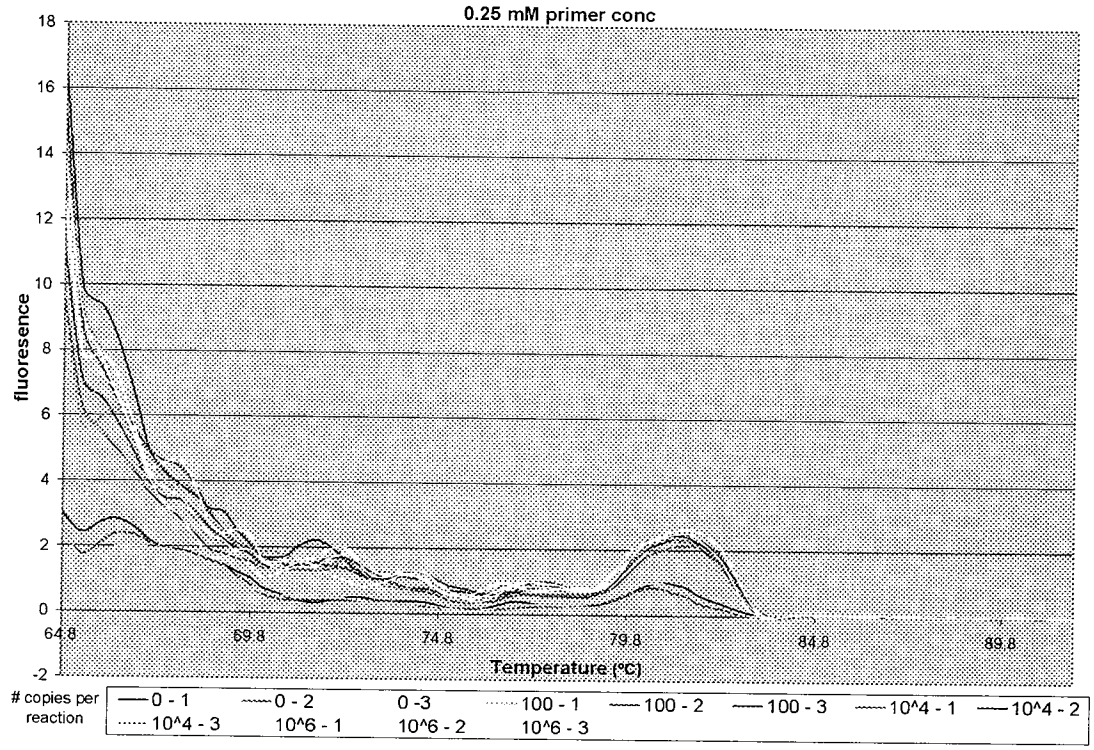


Figure 22: Dissociation curve for PCR performed on 7/7/2008 with 125 nM primer conc.

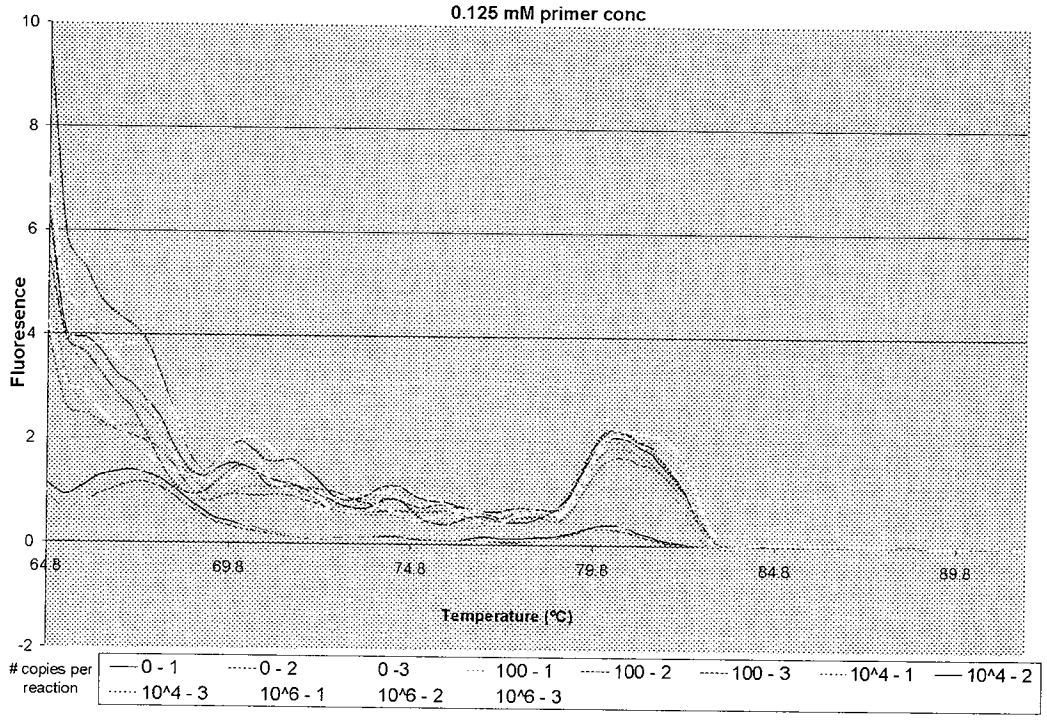
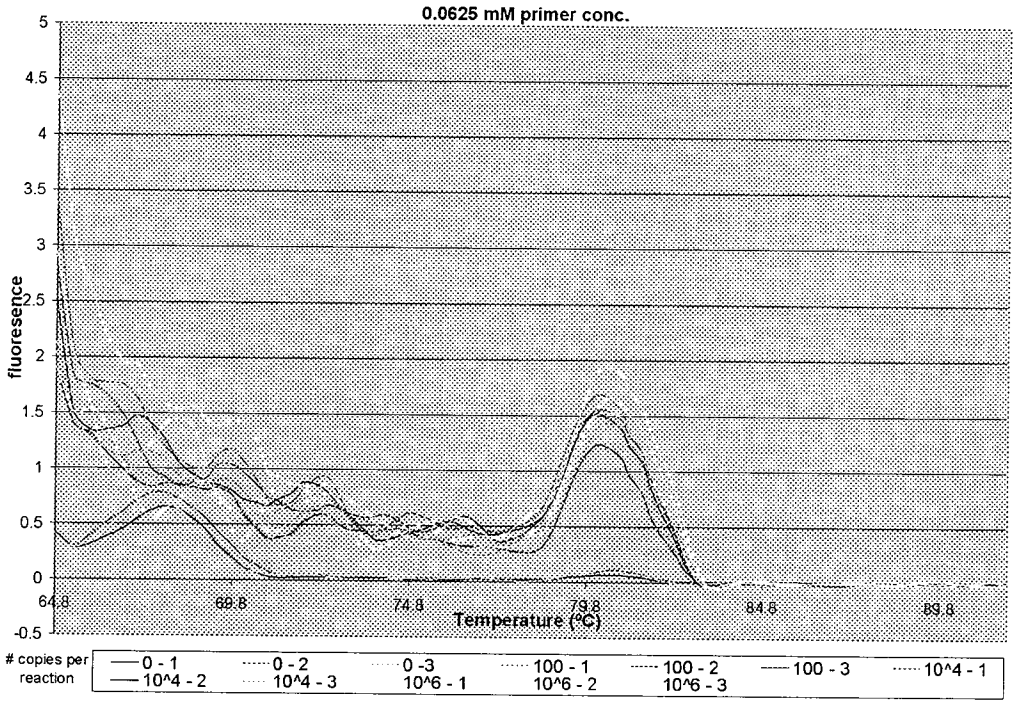


Figure 23: Dissociation curve for PCR performed on 7/7/2008 with 62.5 nM primer conc.



As can be seen in the above figures (Figures 20-23), the largest ratio of primary product to secondary products was seen using the 125 and 62.5 nM concentrations.

In an experiment performed on 7/8/2008 the concentration of forward and reverse primers (LA35F and LA35R) were varied independently. These results indicated that varying the concentration of the individual primers relative to each other did not improve the ratio of product to dimer formed (figures not shown). Having a reverse primer concentration much larger than the forward primer concentration, however, decreased the overall fluorescence of samples, while the Ct values remained about the same. For these reasons and for simplicity, primers were used in equal concentrations for the next set of experiments.

To better define the optimal primer concentration that maximizes product formation and minimizes secondary products, a larger set of control DNA concentrations (poultry marker plasmid) were analyzed at four different primer concentrations; 100 nM, 75 nM, 62.5 nM, and 50 nM. The limit of detections and linearity of their Ct curves and ratio of actual to secondary products in their dissociation curves were compared (Figures 24-27). Note that in these Ct curves the blue points are those above the limit of detection while the pink points are below.

Figure 24: Ct curve for 7/9/2008 with a 100nM primer concentration

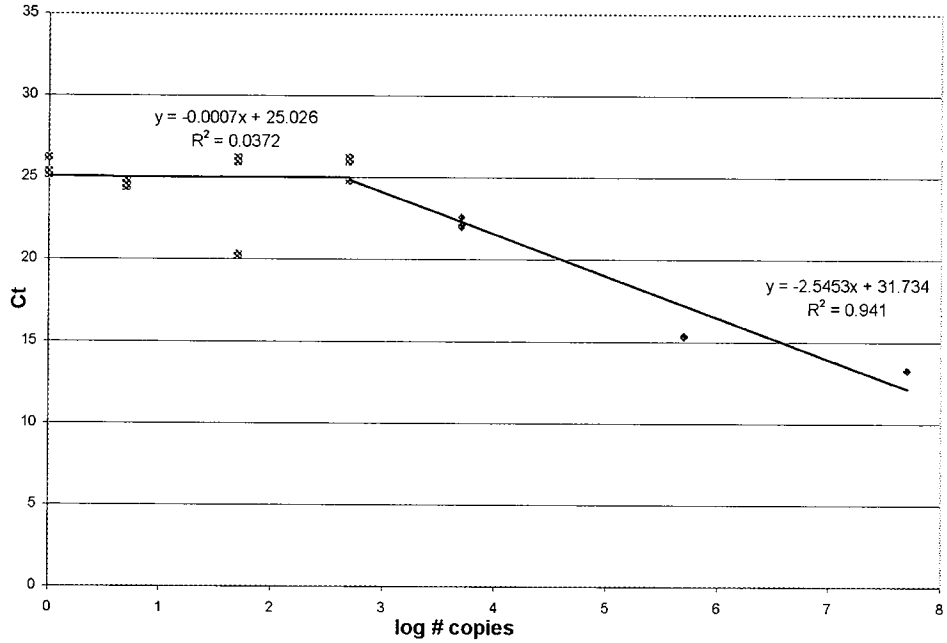


Figure 25: Ct curve for 7/9/2008 with a 75nM primer concentration

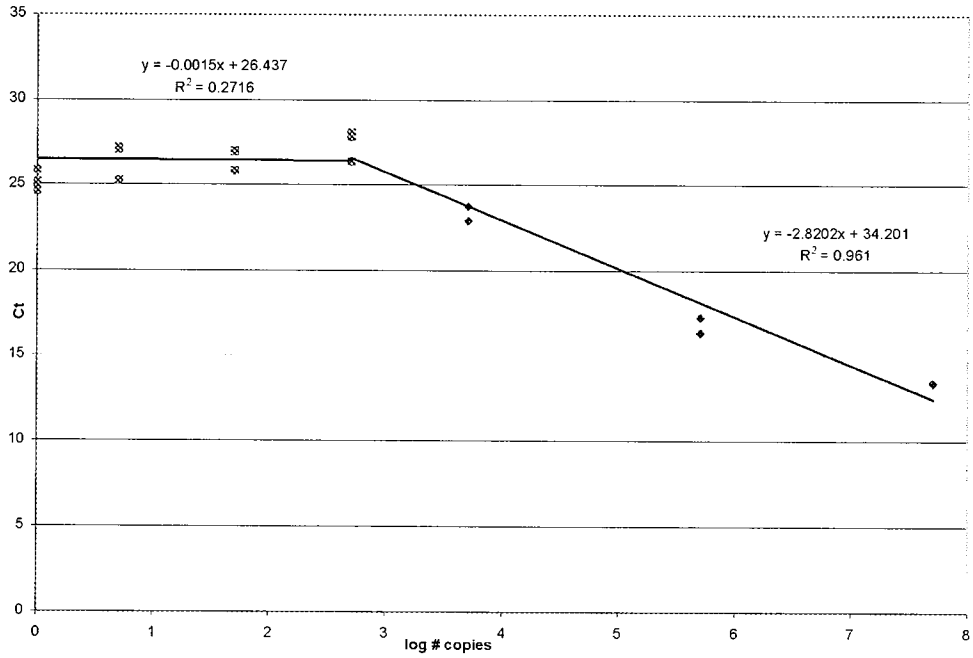


Figure 26: Ct curve for 7/9/2008 with a 62.5 nM primer concentration

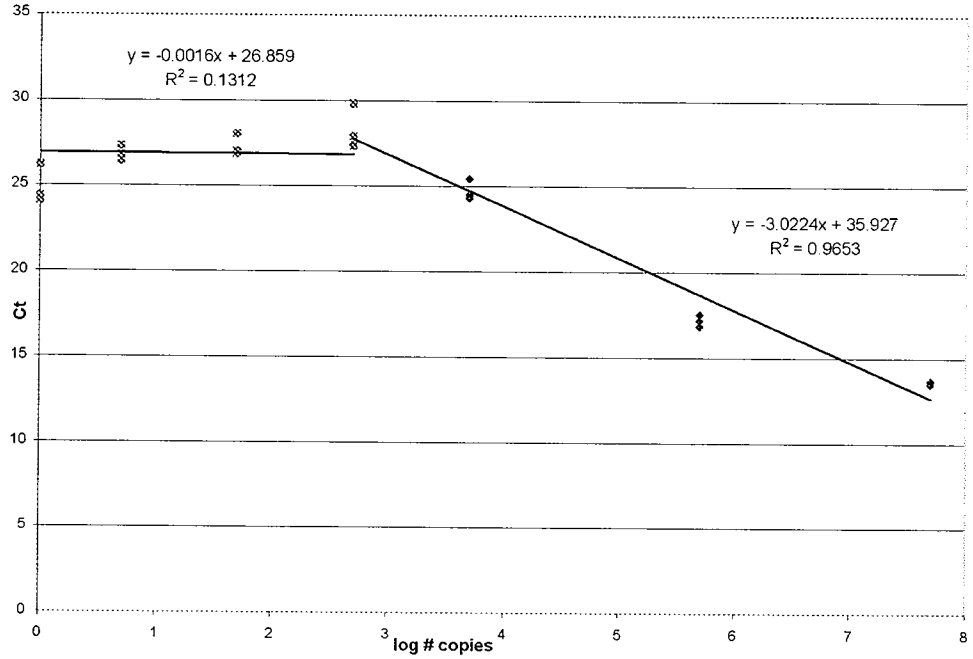
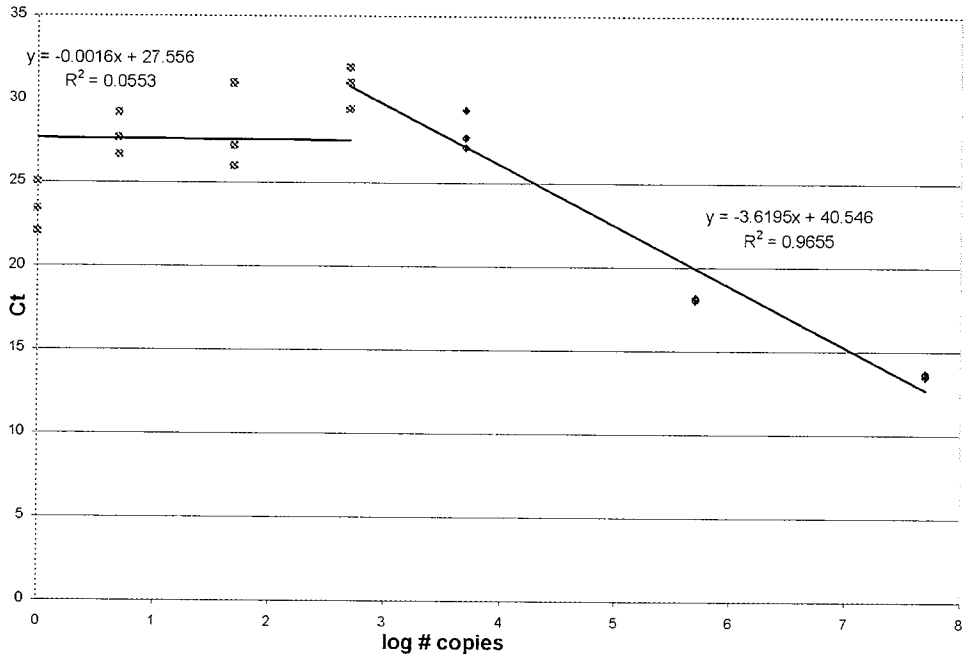


Figure 27: Ct curve for 7/9/2008 with a 50 nM primer concentration



In the results shown in Figures 24-27, the R^2 value and ratio of product to secondary product peaks were used to select the best primer concentration. A primer concentration of 75nM generated a reasonable R^2 value and slope for its standard curve. Based on these results a primer concentration of 75 nM was used for all subsequent studies. However, when dissociation curves for the 62.5 and 75 nM primer concentrations are compared, for high copy number samples the ratio of product to dimer is higher in the 75 nM then the 62.5 nM primer concentration (Figures

28 & 29). Thus there is a trade off in among R^2 values, efficiency of reaction and primer dimer production.

Figure 28: Dissociation curve for 7/9/2008 with a 62.5 nM primer concentration

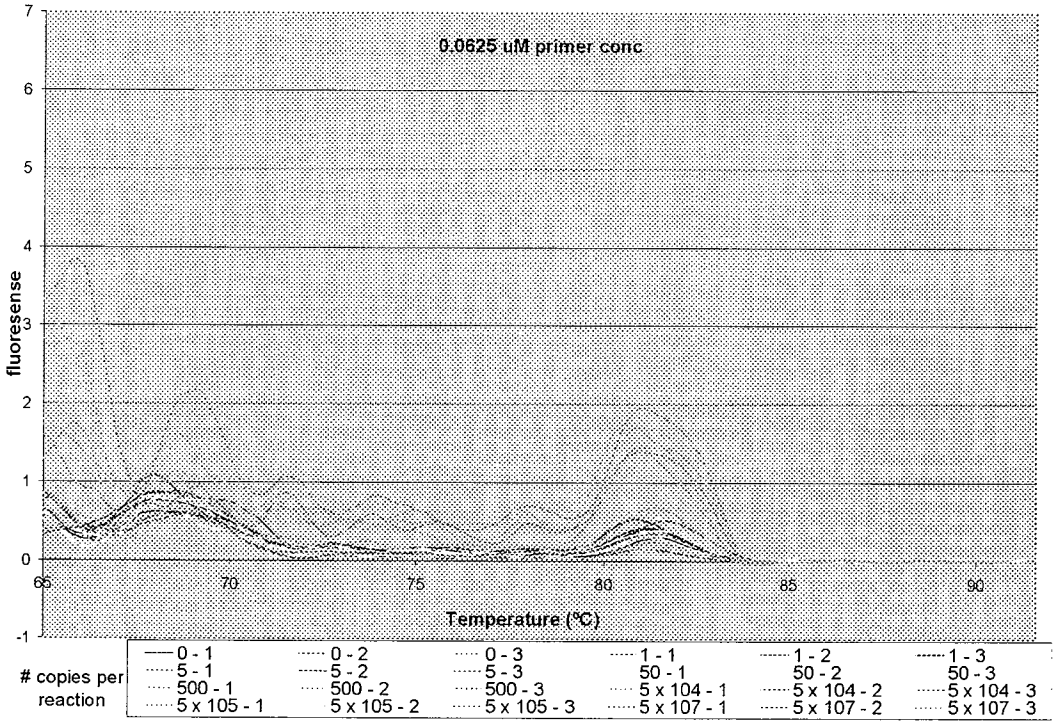
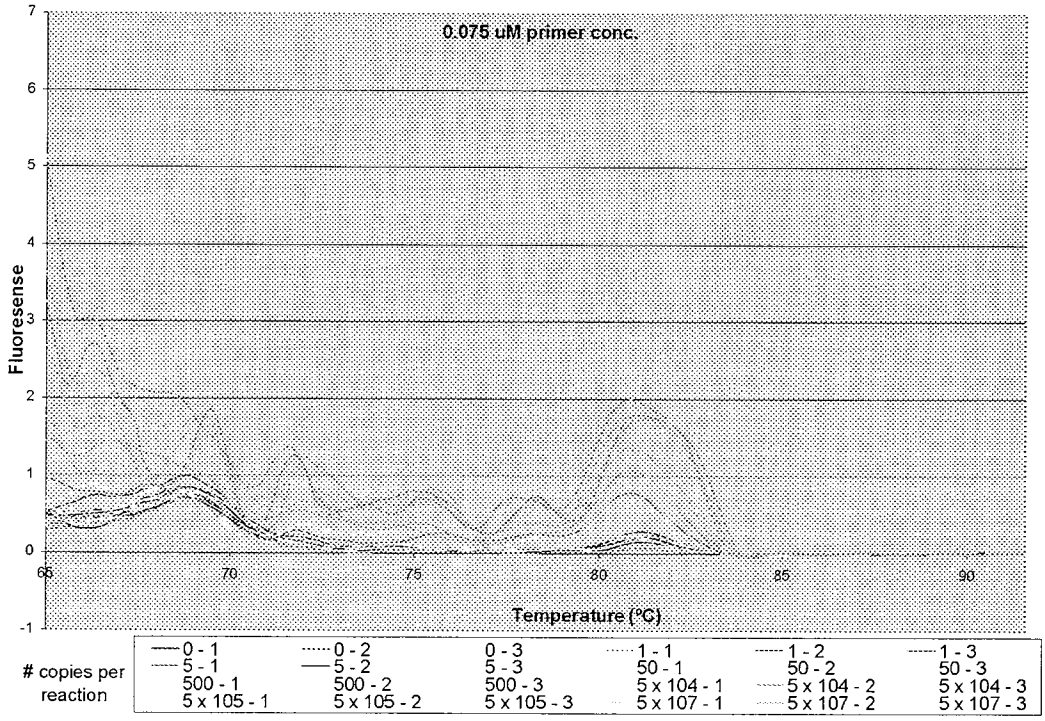


Figure 29: Dissociation curve for 7/9/2008 with a 75 nM primer concentration



VI. Determination of concentration of marker gene in environmental samples.

Based on the above studies, a final protocol was adopted and used to determine concentrations of marker gene in the environmental samples. The total reaction mixture volume was 25 µl, and diluted sample, containing 5 ng of DNA was added in a 5 µl volume. The remaining volume of each reaction consisted of master mix containing 1x FastStart SYBR Green Master Mix with ROX (Roche), 75 nM each of the forward and reverse primers, 5% DMSO and nuclease free water to reach 20 µl.

The thermal cycler protocol started with an incubation of 50°C for 5 minutes, and 95°C for 15 minutes. That was followed by 40 cycles of 30 seconds of dissociation at 95°C, 30 seconds of annealing at 60°C, and 60 seconds of elongation at 75.6°C. After each elongation step the fluorescence of each well was measured. Following amplification there was an incubation at 50°C for 5 minutes, followed by a melting curve step measuring the dissociation of double-stranded DNA between 65°C and 95°C. The qPCR studies were performed on an ABI-Prism 7000 sequence analyzer in optically clear 96 well microplates.

Triplicate reactions were run for all samples along with one matrix spike experiment. In the matrix spike experiment 10^4 copies of control plasmid was added to the reaction. The matrix spike was used to gauge inhibition in samples which were below the threshold for detecting *Brevibacterium*. No sample experienced complete inhibition or an order of magnitude drop in the expected concentration during any of the experiments (Table 2). Therefore inhibition of the qPCR reaction was not a concern with these samples.

To accommodate all of the samples, four sets of plates were run. Each plate had its own separate set of controls. The limit of detection (L.O.D.) was taken to be the point with the highest R^2 value in the Ct versus log copy number curve. The L.O.D. was taken to be 50 copies per reaction. The suggested measure of repeatability was for samples to have a relative standard deviation below 20. Samples where the concentration of each triplicate was below the limit of detection were taken to be negative, independent of the relative standard deviation. Results of these studies are shown in Table 2.

Note that some samples were omitted from the spike analysis due to limited DNA available at the time of the experiment. Omitted reactions are highlighted in yellow.

Table 2: Number of copies of poultry litter marker gene in environmental samples. Results for each replicate are shown in the second and third columns. Note that the assay limit of detection is 50 gene copies per 25 µl reaction.

Sample I.D.	Log (# copies)	# copies	Average # copies	Standard Deviation	Relative St. Dev.
A01	1.704	50.6			
A01	1.611	40.8	52.6	12.9	24.5
A01	1.822	66.4			
A01 + spike (10^4 copies)	4.310	20438.0			
A02	1.498	31.5			
A02	0.375	2.4	14.3	15.2	106.4
A02	0.959	9.1			
A02 + spike (10^4 copies)	4.529	33791.3			
A03	2.323	210.3			
A03	2.247	176.4	206.6	28.5	13.8
A03	2.367	233.1			
A03 + spike (10^4 copies)	4.159	14431.0			
A04	1.557	36.0			
A04	1.526	33.6	34.3	1.5	4.4
A04	1.523	33.3			
A04 + spike (10^4 copies)	3.880	7584.4			
A05	1.530	33.9			
A05	0.828	6.7	22.8	14.2	62.5
A05	1.443	27.7			
A05 + spike (10^4 copies)	3.911	8146.0			
A06	1.619	41.6			
A06	1.597	39.5	37.8	4.8	12.7
A06	1.511	32.4			
A06 + spike (10^4 copies)	4.031	10740.0			
A07	0.366	2.3			
A07	0.371	2.4	2.3	0.0	0.8
A07	0.372	2.4			
A07 + spike (10^4 copies)	2.938	866.5			
A08	1.620	41.6			
A08	1.193	15.6	22.0	17.4	79.0
A08	0.942	8.7			
A08 + spike (10^4 copies)	3.926	8433.4			
A09	1.344	22.1			
A09	1.283	19.2	25.9	9.2	35.6
A09	1.561	36.4			
A09 + spike (10^4 copies)	4.226	16819.7			
A10	1.443	27.7			
A10	1.251	17.8	22.8	5.0	21.8
A10	1.357	22.8			
A10 + spike (10^4 copies)	4.143	13903.0			
A11	1.376	23.8			
A11	1.160	14.5	15.5	7.8	49.9

Sample I.D.	log (# copies)	# copies	Average # copies	Standard Deviation	Relative St. Dev.
A11	0.922	8.4			
A11 + spike (10^4 copies)	3.732	5392.3			
A12	1.773	59.3			
A12	1.673	47.1	56.7	8.5	15.0
A12	1.803	63.5			
A12 + spike (10^4 copies)	4.049	11192.4			
A13	1.585	38.5			
A13	1.792	61.9	71.8	39.3	54.7
A13	2.061	115.2			
A13 + spike (10^4 copies)	4.075	11893.8			
A14	2.091	123.2			
A14	1.958	90.9	72.1	62.6	86.7
A14	0.374	2.4			
A14 + spike (10^4 copies)	4.245	17586.1			
A15	1.683	48.2			
A15	1.765	58.2	46.6	12.6	27.0
A15	1.522	33.2			
A15 + spike (10^4 copies)	3.748	5601.4			
A16	0.921	8.3			
A16	0.873	7.5	16.2	14.4	88.8
A16	1.516	32.8			
A16 + spike (10^4 copies)	3.778	5998.4			
A17	0.509	3.2			
A17	1.462	29.0	14.0	13.4	95.4
A17	0.993	9.8			
A17 + spike (10^4 copies)	3.490	3090.9			
A18	0.374	2.4			
A18	0.863	7.3	4.6	2.5	54.8
A18	0.606	4.0			
A18 + spike (10^4 copies)	3.241	1743.2			
A19	0.824	6.7			
A19	0.827	6.7	6.7	0.0	0.4
A19	0.825	6.7			
A19 + spike (10^4 copies)	4.107	12782.7			
A20	0.827	6.7			
A20	0.826	6.7	6.7	0.0	0.2
A20	0.827	6.7			
A20 + spike (10^4 copies)	4.289	19438.7			
A21	0.831	6.8			
A21	0.826	6.7	6.7	0.0	0.7
A21	0.828	6.7			
A21 + spike (10^4 copies)	4.232	17048.8			

Sample I.D.	log (# copies)	# copies	Average # copies	Standard Deviation	Relative St. Dev.
A22	0.825	6.7			
A22	0.831	6.8	6.7	0.050515	0.7494
A22	0.830	6.8			
A22 + spike (10^4 copies)	4.050	11214.3			
A23	0.827	6.7			
A23	0.820	6.6	6.7	0.074283	1.1145
A23					
A23 + spike (10^4 copies)					
A24	0.828	6.7			
A24	0.830	6.8	6.7	0.016676	0.2473
A24	0.828	6.7			
A24 + spike (10^4 copies)	4.187	15382.1			
A25	0.826	6.7			
A25	0.830	6.8	6.7	0.047846	0.7135
A25	0.824	6.7			
A25 + spike (10^4 copies)	4.073	11820.9			
A26	0.830	6.8			
A26	0.823	6.7	6.7	0.054846	0.8193
A26	0.824	6.7			
A26 + spike (10^4 copies)	3.959	9101.8			
A27	0.831	6.8			
A27	0.827	6.7	6.7	0.045721	0.6799
A27	0.825	6.7			
A27 + spike (10^4 copies)	4.366	23250.1			
A28	0.827	6.7			
A28	0.829	6.7	6.7	0.014346	0.2134
A28	0.827	6.7			
A28 + spike (10^4 copies)	3.697	4982.5			
A29	0.828	6.7			
A29	0.824	6.7	6.7	0.030765	0.4589
A29	0.826	6.7			
A29 + spike (10^4 copies)	4.063	11557.7			
A30	0.825	6.7			
A30	0.828	6.7	6.7	0.017874	0.2664
A30	0.827	6.7			
A30 + spike (10^4 copies)	4.051	11234.0			
A31	0.823	6.7			
A31	0.827	6.7	28.7	38.15191	132.89
A31	1.862	72.8			
A31 + spike (10^4 copies)					
A32	2.063	115.5			
A32	1.624	42.0	63.0	45.76683	72.631
A32	1.498	31.5			
A32 + spike (10^4 copies)	3.812	6491.8			
	log		Average	Standard	Relative

Sample I.D.	(# copies)	# copies	# copies	Deviation	St. Dev.
B01	3.598	3965.8			
B01	3.638	4347.1	3985.8	351.6776	8.8232
B01	3.562	3644.6			
B01 + spike (10^4 copies)	4.034	10821.8			
B02	3.176	1501.1			
B02	3.061	1151.1	1397.0	213.7341	15.3
B02	3.187	1538.6			
B02 + spike (10^4 copies)	3.788	6140.2			
B03	3.382	2412.0			
B03	3.505	3198.6	2328.7	914.4405	39.269
B03	3.138	1375.4			
B03 + spike (10^4 copies)	3.746	5575.1			
B04	3.999	9981.4			
B04	4.002	10047.2	9890.2	217.5678	2.1998
B04	3.984	9641.8			
B04 + spike (10^4 copies)	4.211	16271.9			
B05	4.729	53595.9			
B05	4.645	44143.2	39895.9	16245.52	40.72
B05	4.341	21948.6			
B05 + Spike (10^4 copies)	4.942	87401.5			
B06	4.452	28332.6			
B06	4.149	14107.4	17322.7	9805.86	56.607
B06	3.979	9528.2			
B06 + Spike (10^4 copies)	4.530	33881.3			
B07	4.127	13391.7			
B07	4.401	25202.4	73005.5	93213.11	127.68
B07	5.256	180422.6			
B07 + Spike (10^4 copies)	4.820	66065.5			
B08	4.439	27502.9			
B08	4.914	82018.9	60247.1	28866.8	47.914
B08	4.853	71219.4			
B08 + Spike (10^4 copies)	4.719	52349.3			
B09	4.703	50487.6			
B09	4.692	49233.6	50619.7	1456.582	2.8775
B09	4.717	52137.8			
B09 + Spike (10^4 copies)	4.839	69029.6			
B10	4.593	39150.7			
B10	4.949	88942.6	57292.0	27507.84	48.013
B10	4.641	43782.8			
B10 + Spike (10^4 copies)	4.654	45086.1			

Sample I.D.	log (# copies)	# copies	Average # copies	Standard Deviation	Relative St. Dev.
B11	4.652	44850.4			
B11	4.181	15169.7	24505.2	17639.3	71.982
B11	4.130	13495.6			
B11 + Spike (10^4 copies)	4.737	54631.3			
B12	4.622	41859.4			
B12	4.519	33026.3	36683.3	4608.283	12.562
B12	4.546	35164.3			
B12 + Spike (10^4 copies)	4.927	84434.6			
B13	4.837	68759.0			
B13	4.177	15031.8	40338.6	26998.61	66.93
B13	4.571	37224.9			
B13 + Spike (10^4 copies)	4.639	43578.5			
C01	0.347	2.2			
C01	0.344	2.2	2.2	0.011816	0.5332
C01					
C01 + Spike (10^4 copies)					
C02	1.339	21.8			
C02	1.737	54.5	72.9	62.24227	85.436
C02	2.153	142.2			
C02 + Spike (10^4 copies)	4.642	43867.9			
C03	1.948	88.8			
C03	1.940	87.2	59.4	49.52184	83.371
C03	0.347	2.2			
C03 + Spike (10^4 copies)	4.124	13318.4			
C04	0.829	6.7			
C04	1.844	69.8	42.4	32.33893	76.196
C04	1.706	50.8			
C04 + Spike (10^4 copies)	4.303	20111.3			
C05	1.738	54.6			
C05	1.444	27.8	42.2	13.51277	32.039
C05	1.644	44.1			
C05 + Spike (10^4 copies)	3.663	4600.3			
C06	1.076	11.9			
C06	1.236	17.2	10.4	7.618552	72.965
C06	0.342	2.2			
C06 + Spike (10^4 copies)	4.009	10201.1			
C07	0.344	2.2			
C07	1.340	21.9	8.8	11.35134	129.57
C07	0.344	2.2			
C07 + Spike (10^4 copies)	4.292	19566.6			
C08	1.139	13.8			
C08	0.347	2.2	6.1	6.673284	109.98
C08	0.344	2.2			
C08 + Spike (10^4 copies)	3.527	3363.1			
	log		Average	Standard	Relative

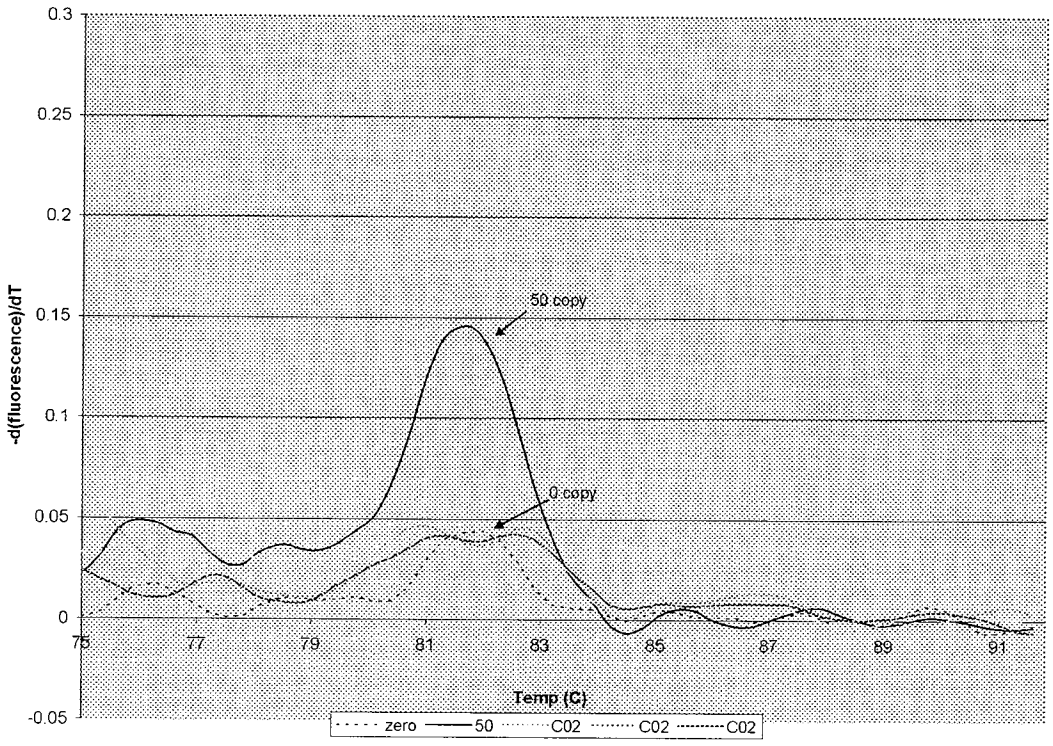
Sample I.D.	(# copies)	# copies	# copies	Deviation	St. Dev.
C09	0.347	2.2			
C09	0.348	2.2	2.2	0.002824	0.1269
C09	0.347	2.2			
C09 + Spike (10^4 copies)	3.688	4873.8			
C10	0.683	4.8			
C10	0.672	4.7	4.8	0.061345	1.2898
C10	0.676	4.7			
C10 + Spike (10^4 copies)	3.527	3361.3			
C11	1.053	11.3			
C11	1.532	34.0	16.7	15.37415	92.112
C11	0.676	4.7			
C11 + Spike (10^4 copies)					
C12	1.782	60.6			
C12	1.530	33.9	56.4	20.73063	36.758
C12	1.873	74.7			
C12 + Spike (10^4 copies)					
C13	0.670	4.7			
C13	0.675	4.7	4.7	0.035073	0.7434
C13	0.676	4.7			
C13 + Spike (10^4 copies)	3.933	8563.1			
C14	0.674	4.7			
C14	0.674	4.7	4.7	0.022421	0.4735
C14	0.678	4.8			
C14 + Spike (10^4 copies)	4.165	14610.7			
C15	1.389	24.5			
C15	1.160	14.5	15.2	8.88461	58.314
C15	0.830	6.8			
C15 + Spike (10^4 copies)	3.584	3841.1			
C16	0.672	4.7			
C16	0.674	4.7	4.7	0.016378	0.3469
C16	0.675	4.7			
C16 + Spike (10^4 copies)	4.208	16126.6			
C17	0.680	4.8			
C17	0.682	4.8	4.8	0.018668	0.3901
C17	0.678	4.8			
C17 + Spike (10^4 copies)	4.215	16391.9			
C18	1.171	14.8			
C18			14.8	-	-
C18					
C18+ Spike (10^4 copies)					

*Note: Well highlighted in yellow were omitted due to lack of sufficient DNA in samples.

Results in Table 2 show that poultry litter biomarker gene copy number measured in some of the sample replicates were quite variable, so that while some were above the limit of detection others were below the assay's limit of detection. These samples included: A01, A12, A13, A14, A15, A32, C02, C03, C04, C05, C08, and C12. For these samples, the melting curve was analyzed in the region between 75°C and 91°C, which corresponds to the melting temperature of the primary product (81°C).

On each melting curve (Figures 30-34) the limit of detection for all reactions was 50 copies per reaction. For a sample to be considered positive for the poultry marker, the melting curve for the PCR product in that region had to be greater than that seen in the non-template control and greater than the melting curve generated by the 50 copy positive control (the LOD). If a sample's fluorescence was below that of the zero copy (negative control) samples, it was considered negative for the presence of the poultry marker gene. Moreover, samples producing maximum fluorescence below that produced in the 50 copy reaction were considered below our limit of detection and reported as negative for the marker gene. All samples producing melting curves with fluorescence at or above the 50 copy per reaction LOD were considered positive. An example of a negative reaction is shown in Figure 30.

Figure 30: Melting Curve for Sample C02



Samples A01, A12, A13, A14, A15, A31 and A32 all were below the zero copy per reaction standard, samples C02, C03, C05, and C08 all had at least one sample which produced curves above that produced by zero copy per reaction and below that produced by the 50 copy reaction. Taken together, all of these samples were negative for the presence of the poultry litter marker gene. In addition, samples C04 and C12 both had one replicate with curves above the peak for the 50 copy per reaction standard, and had their other two replicates between the zero and 50

copy standards (Figure 31-32). These sampled may be positive for the poultry litter marker gene, but have copy numbers near the limit of detection.

Figure 31: Melting Curve for Sample C04

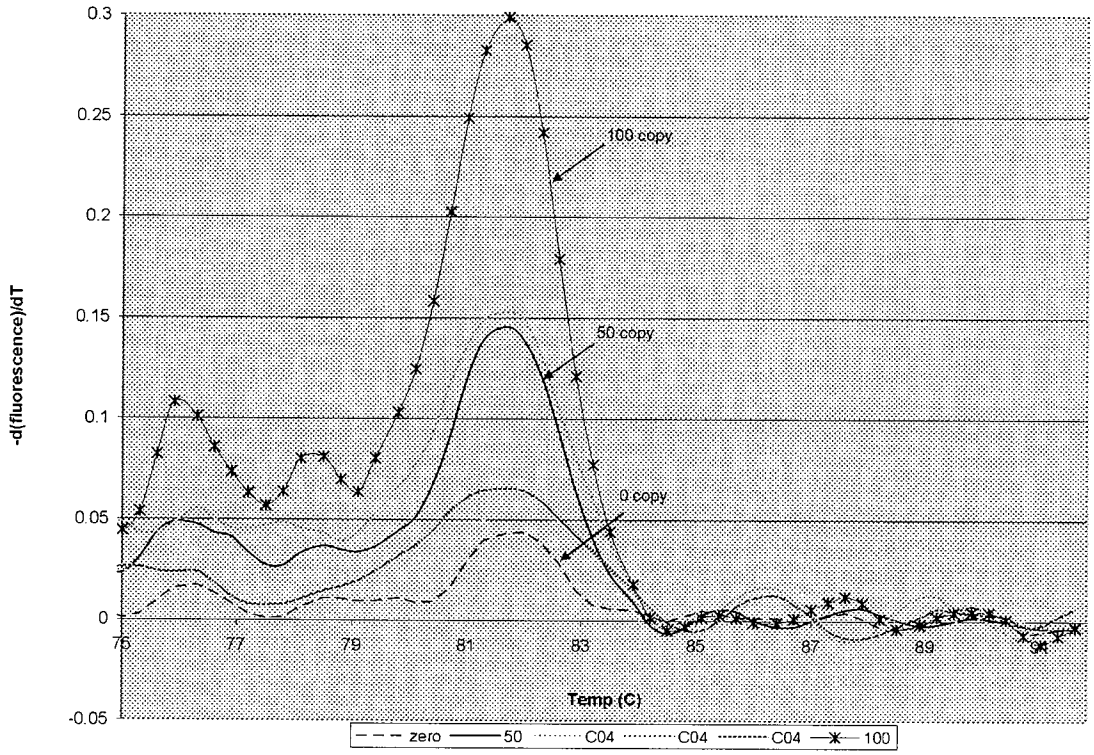
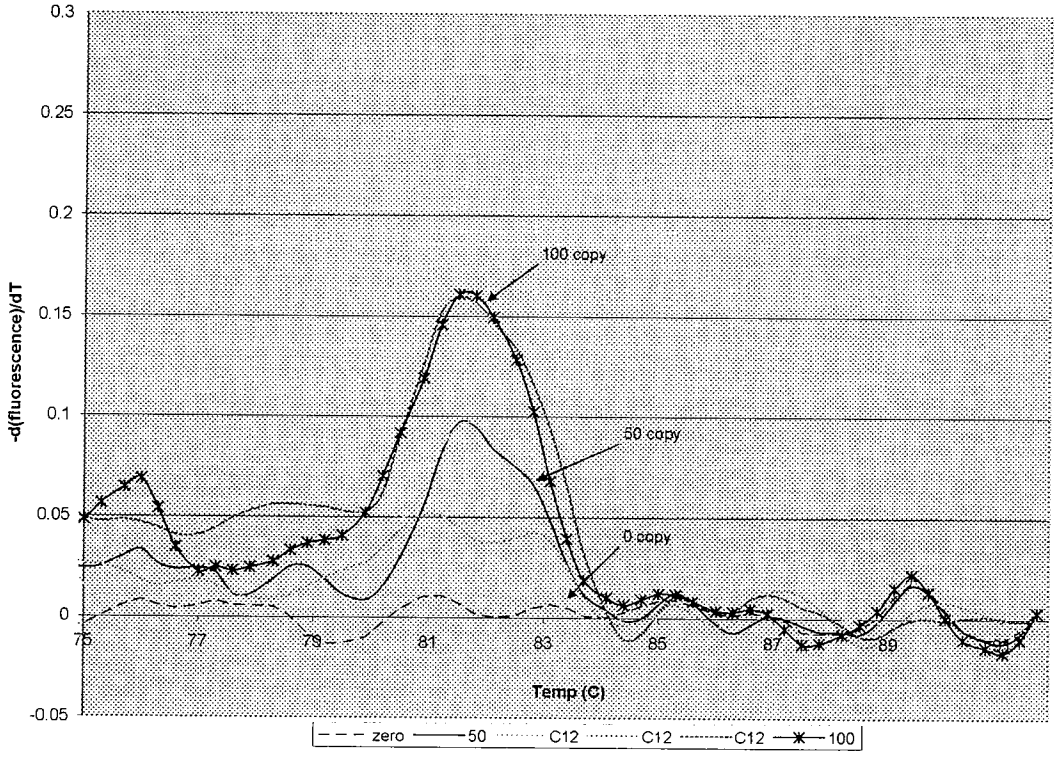


Figure 32: Melting Curve for Sample C12



To more accurately determine if samples C04 and C12 were positive or negative for the presence of the poultry-specific marker gene a second qPCR experiment was performed. We also wished to rerun sample A03 which was above the limit of detection, but was thought not to have the marker gene. However, due to low concentrations of DNA in our sample tubes, we requested new environmental samples from North Wind. Stocks of the original samples A03, C08 & C12 were received by us on 9/30/2008. However, since there were no stocks available for C04 (no sample left at North Wind), samples C23, C24, and C25 were sent to us by North Wind (received on 9/31/2008) and acted as proxies for the missing samples. These proxy samples were extracted from the same lot of material but were not included in the original set sent to us. The qPCR was performed using the same conditions as described previously.

Table 3: Number of copies of marker gene in environmental samples.

Sample ID	log (# copies)	# copies	Sample ID	log (# copies)	# copies
A03 (4/11/08)	1.77	59.4	C23	-0.18	0.7
A03 (4/11/08)	-0.18	0.7	C23	2.27	186.2
A03 (4/11/08)	-0.17	0.7	C23	-0.18	0.7
A03 (4/11/08) + SPIKE	4.19	15501.4	C23 + SPIKE	3.58	3802.9
A03 (9/29/08)	-0.20	0.6	C24	-0.19	0.6
A03 (9/29/08)	-0.18	0.7	C24	-0.18	0.7
A03 (9/29/08)	-0.18	0.7	C24	0.66	4.6
A03 (9/29/08) + SPIKE	3.93	8435.5	C24 + SPIKE	4.06	11487.0
C08	-0.18	0.7	C25	-0.18	0.7
C08	-0.18	0.7	C25	-0.18	0.7
C08	-0.18	0.7	C25	-0.17	0.7
C08 + SPIKE	4.13	13575.3	C25 + SPIKE	3.90	7883.8
C12	-0.17	0.7			
C12	0.10	1.3			
C12	-0.18	0.7			
C12 + SPIKE	4.21	16074.8			

Results of these studies (Table 3) showed that all of the retested samples were below the limit of detection, except samples A03 and C23, which had one replicate showing a small amount of the marker gene. The dissociation curves for both samples were analyzed (Figures 33 and 34). The one triplicate of A03 which was above the limit of detection had the majority of its fluorescence originating from the secondary product portion of the dissociation curve, thus this sample is viewed as being negative for the *Brevibacterium* marker gene.

The one replicate of sample C23, which had fluorescence above the limit of detection, had the majority of its signal coming from from the primary PCR product portion of the dissociation curve (Figure 34). The fluorescence peak at 80°C was greater than that produced using the 200 copies per reaction control sample. These results suggest that there is some of the target gene in this sample, although it at a very low concentration.

Figure 33: Dissociation curve of sample A03

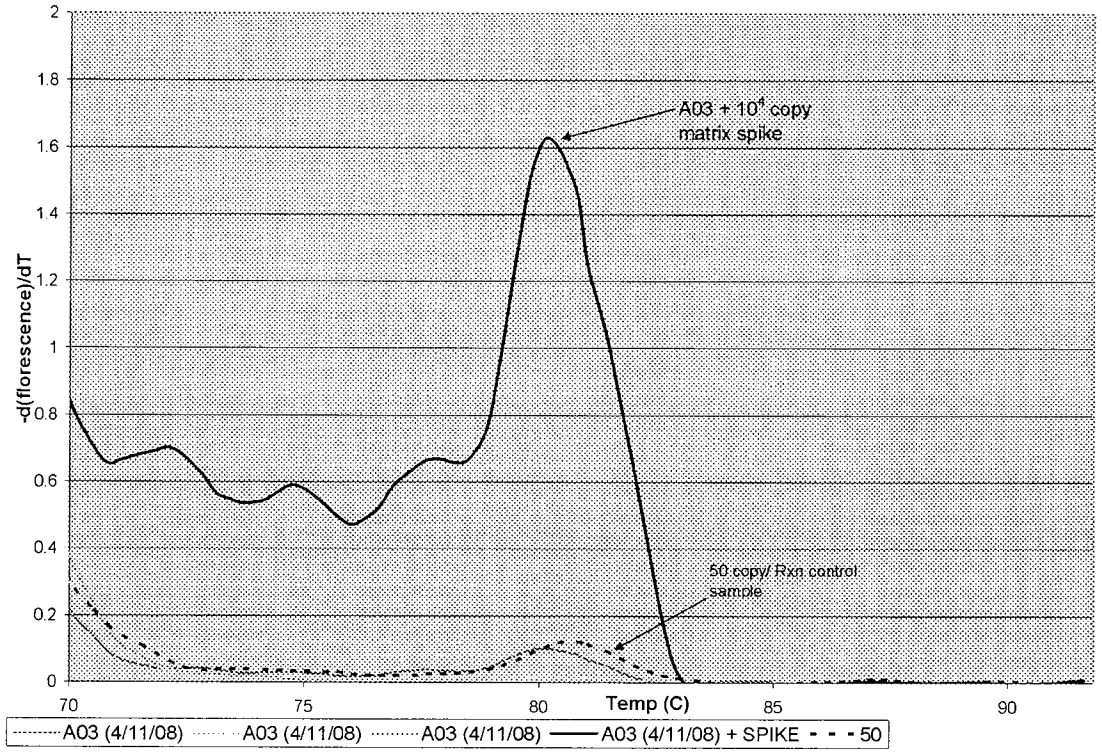
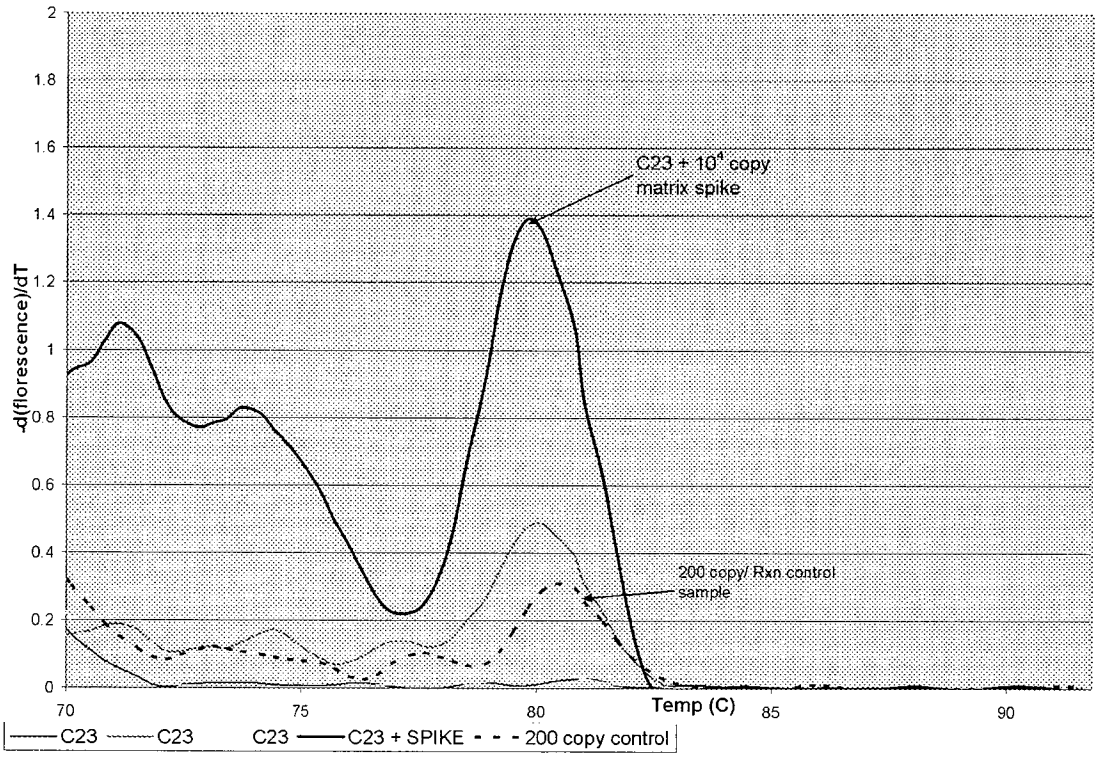


Figure 34: Dissociation curve of sample C23



VII. Summary

Based on the above studies, a final protocol has been adapted and used to determine concentrations of a *Brevibacterium*-based poultry marker gene in the environmental samples. The reaction mixture (25 µl) contains master mix containing 1x FastStart SYBR Green Master Mix with ROX (Roche), 75 nM each of the forward and reverse primers, 5% DMSO, a diluted sample containing 5 ng of DNA in a 5 µl volume, and nuclease free water to reach 20 µl final volume.

The thermal cycler protocol used was as follows: 50°C for 5 min, and 95°C for 15 min, followed by 40 cycles of 30 sec of dissociation at 95°C, 30 sec of annealing at 60°C, and 60 sec of elongation at 75.6°C. For qPCR, fluorescence of each well is measured after each elongation step. Following amplification there was an incubation at 50°C for 5 minutes, followed by a melting curve experiment measuring the dissociation of samples between 65°C and 95°C. The qPCR studies were performed on an ABI-Prism 7000 sequence analyzer in optically clear 96 well microplates. Based on qPCR, the protocol adopted had a limit of detection of 50 copies per 25 µl reaction volume.

The entire set of "B" samples were positive for the presence of the marker gene, and this gene was present in high concentration, in the order of tens of thousands of copies of the marker gene per 5 ng of sample DNA. Thus, these samples contained the marker gene indicative of contamination with poultry litter or poultry feces.

The following samples were below the limit of detection for the Brevibacterium marker gene that is stated to be indicative of poultry litter contamination: C01, C06, C07, C08, C09, C10, C11, C13, C14, C15, C16, C17, C18, C23, C24 and C25 (proxies for sample C04). Two of three replicate analyses of samples C02, C03, C04, C05, and C12 were positive for the presence of the marker gene, and the average of all three replicates were quantitatively above the limit of detection. Consequently, these samples were viewed as containing the marker gene.
The entirety of set "A" was below the limit of detection and was considered negative for the presence of the marker gene. Thus, these samples likely were likely not contaminated with poultry litter or poultry feces.